INTERVIEW SUMMARY

Applicants wish to thank Examiner Cathy Worley for the courtesy of a telephonic interview held on March 26, 2007. During this interview, the §102(e) rejection and declaration of Dr. Kitto were discussed. Applicants noted that the lab notebook page entitled "Preparation of extracts for toxicity" specifically refers to "max LDH." Applicants also noted that the declaration clearly indicates in points (3) and (4) that the datasheets represent work done using LDH-C4 expressed in plants. After speaking with her supervisor, examiner Worley telephoned the undersigned and indicated that the §102(e) rejection would be withdrawn.

REMARKS

Status of the Claims

Claims 1-6 are currently pending and claims 7-15 are withdrawn. Claim 5 has been amended herein. Support for the amendment can be found in the specification (*e.g.*, p. 16-19). Claims 16 and 17 have been added. Support for these claims can be found throughout the specification and the originally filed claims. No new matter has been added.

Method claims 7-8 incorporate the limitations of claim 1, which are within the scope of Group I of the Restriction Requirement. Therefore, these method claims are specifically related to the elected product claims of Group I as product and method of use and should be rejoined in the case accordingly. Applicants therefore respectfully request rejoinder and consideration of claims 7-8 on this basis.

Response to Rejection Under §102(e)

The Action rejects claims 1 and 6 under §102(e) as anticipated anticipated by Kirk *et al.* As stated above in the interview summary, Examiner Worley has indicated to Applicants that this rejection will be withdrawn.

Response to Rejection Under §103

The Action rejects claims 1-6 under §103 as unpatentable over Bleil *et al.* in view of Goldberg *et al.* In response, Applicants respectfully traverse.

The argument in the Action appears to be based on an "obvious to try" rationale, which the Federal Circuit has specifically held is insufficient to support a rejection under §103. The Action has not demonstrated that there would be any guarantee that an LDH-C4 expressed in a plant would form an immunogenic LDH-C4. Additionally, the Action has not provided any indication that administration (*e.g.*, oral administration) of an LDH-C4 protein expressed in a plant would result in an immunocontraceptive immune response. Such an expectation of success must be present and established on the record in order to maintain an obviousness rejection. *In re Vaeck*, 947 F.2d 488, 20 USPQ 2d 1438 (Fed. Cir. 1991), *see also*, M.P.E.P. § 2142. The rejection must further be supported by "substantial evidence" in accordance with the Administrative Procedure Act ("APA"). *See In re Gartside*, 203 F.3d 1305, 1314-15 (Fed. Cir. 2000); 5 U.S.C. § 706(A), (E), 1994; *see also In re Zurko,* 59 USPQ 2d 1693 (Fed. Cir. 2001).

Applicants note that there is presently a significant degree of uncertainty in the art regarding immune responses to orally administered proteins or peptides (*e.g.*, Feldman et al. 2005; Hogan et al. 2000, provided herewith). Further, applicants note that oral administration of a protein or peptide to an animal can sometimes induce an immune response which is **opposite** to

the immune response observed in the instant application. For example, Strober (2004, provided herewith for the convenience of the Examiner) describes **tolerance** resulting from oral administration of an antigen.

In contrast, the present application provides evidence, *e.g.*, in Example 10, that oral administration of a transgenic plant expressing an LDH-C4 protein can generate an immunocontraceptive immune response. Even if it is assumed, *arguendo*, that it was obvious to try to express an LDH-C4 in a plant, the pharmaceutical properties of the instantly claimed genetically modified plant are nonobvious (*e.g.*, oral administration of the plant to an animal can induce an immunocontraceptive response). Evidence of a new property demonstrates nonobviousness. ***In re Papesch***, 315 F.2d 381, 137 U.S.P.Q. 43 (CCPA 1963).

The Action has failed to provide a *prima facie* case of obviousness for the reasons as follows. Specifically, the Action has not taught all of the claim limitations, as required by MPEP§2143. Further, the Action has not demonstrated motivation to combine Bleil with Goldberg.

The Action has not taught all of the claim limitations, as required by MPEP§2143.03. Specifically, the Action has not shown in the prior art the concept of expressing an LDH-C4 in a plant. The Action has not shown in the prior art the concept of expressing an antigenic fragment of a protein in a plant. Further, the Action has not shown in the prior art the concept of expressing an immunocontraceptive antigenic fragment of a protein in a plant. Finally, the Action has not shown in the prior art the concept of expressing an immunocontraceptive antigenic fragment of an LDH-C4 in a plant. Applicants agree with the Examiner, as stated in the Office Action of 26 May 2006, that Bleil does not teach LDH-C4.

The Action has also not provided motivation to combine Bleil with Goldberg. Again, Applicants agree with the Examiner, as stated in the Office Action of 26 May 2006, that Bleil does not teach LDH-C4. Applicants note that Bleil does not teach the expression of ***all*** immunocontraceptives in a plant, but rather that Bleil relates ***specifically*** to the sp56 antigen.

In particular, Applicants have evaluated Bleil and have only found one reference to LDH-C4 (p. 2, lines 27-28), among several other antigens, which is subsequently used to ***differentiate*** these antigens from sp56, as stated in Bleil:

> **In contrast** to the above-noted sperm antigens, however, sp56 is **unique** in the following respects... **In view of the foregoing characteristics of sp56**, it is **therefore** desirable to design an immunocontraceptive vaccine based on a sp56 antigenic polypeptide. (emphasis added, p.4, lines 6-17)

Implicit with these statements is the indication that the other antigens would not be desirable to use in Bleil because they do not possess the characteristics of sp56. Bleil thus not only fails to provide motivation to use other immunocontraceptives, but also **teaches away** from using the other antigens.

Applicants have reviewed Bleil, and have not been able to identify any section relating to expressing an antigenic fragment of an immunocontraceptive in a plant. Applicants note that the Action has not provided such evidence in Bleil. As stated in MPEP §2142, "The examiner bears the initial burden of factually supporting any prima facie conclusion of obviousness."

Goldberg does not provide motivation to express an antigenic peptide in a plant The Action has not provided and Applicants have been unable to find any indication in Goldberg relating to transgenic expression in plants. The Action has not shown that the mere indication that a region of a protein may be antigenic relates to expression in a transgenic plant. The Action

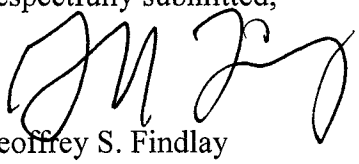
has not shown that Goldberg provides any indication or motivation to express an immunocontraceptive in a plant. Nor has the Action shown Goldberg provide any indication or motivation to express an antigenic fragment of an immunocontraceptive in a plant. As noted by the Court of Appeals for the Federal Circuit, "[a] person of ordinary skill in the art is also presumed to be one who thinks along the lines of conventional wisdom in the art and is not one who undertakes to innovate, whether by patient, and often expensive, systematic research or by extraordinary insight; it makes no difference which." *The Standard Oil Company v. American Cyanamid Co.*, 227 U.S.P.Q. 293 (Fed. Cir. 1985) (emphasis supplied). Applicants submit that this rejection may be based upon hindsight reconstruction, which is impermissible as stated in MPEP §2141.01.

Withdrawal of the rejection is thus respectfully requested.

Conclusion

In light of the foregoing, Applicants submit that all claims are in condition for allowance, and an early indication to that effect is earnestly solicited. The Examiner is invited to contact the undersigned at (512) 536-5674 with any questions, comments or suggestions relating to the referenced patent application.

Respectfully submitted,



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Date: April 11, 2007

NATURE|Vol 435|2 June 2005|doi:10.1038/nature03727

Design of effective immunotherapy for human autoimmunity

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A better understanding of the molecules involved in immune responses has identified many potential targets for the treatment of autoimmune diseases. But although successful therapies have been found for immune disorders in animal studies, few have passed the much harder test of treating human diseases. So far, non-antigen-specific approaches, such as the blocking of tumour-necrosis factor, are achieving some success but the same is not true for antigen-specific approaches. Future therapies will probably include both non-antigen-specific strategies that target cytokines (cell–cell signalling molecules) or block the molecules that stimulate immune responses, and antigen-specific therapies that induce tolerance to self antigens.

Immunotherapy is a type of treatment that uses immunological tools, such as monoclonal antibodies, receptor–immunoglobulin fusion proteins, vaccines and immune cells. Such therapeutic options have only been available in the past 10 to 15 years, owing to major advances in medical science and technology, but are now increasingly being used to tackle a wide spectrum of human diseases. The application of immunotherapy to autoimmune diseases is broadening our understanding of the human immune response, with responses to treatment providing unique insights into pathological mechanisms. The availability of effective immunosuppressive drugs[1] to ameliorate the immune-mediated rejection of transplants contrasts sharply with the paucity of drugs that successfully treat autoimmune diseases. This implies that whereas a transplant is a classic acute challenge to an otherwise normal immune system, chronic autoimmune diseases are somehow different.

The failure of most immunological approaches that are effective in animal models[2,3] to modulate autoimmune disease in humans suggests that we do not understand many of the principles behind the pathogenic mechanisms of these diseases. We remain ignorant of what drives the chronicity of these conditions, which can last for decades, and of how we can normalize the immune and pro-inflammatory responses once they commence. The rate-limiting steps of the early immune response (such as the presentation of antigen by dendritic cells, the expansion of $CD4^+$ helper T-cell populations and the induction of costimulatory-molecule expression) may not be rate limiting or critical for the chronic phase of the disease and the resultant tissue destruction, which often occur years after onset.

Human transplants, which often undergo chronic rejection[1] despite continuous immunosuppressive therapy and early success, have confirmed our lack of understanding of chronicity. Furthermore, results in acute animal models of autoimmunity are often not predictive for the treatment of chronic human immune disorders[2–4]. Because we do not understand the differences between the chronic and acute response, we cannot be sure which, if any, animal models of disease provide good reflections of the key processes that occur in human disease. A further complication for the transition from animal to human studies is the necessary preoccupation with safety in human immunotherapy, a relatively ignored issue in animal models.

Here, we highlight recent successes in immunotherapy, which is now benefiting almost a million patients with chronic diseases, such as rheumatoid arthritis and Crohn's disease, that are unresponsive to other treatments. We contrast the effectiveness of therapies aimed at inhibiting the non-antigen-specific pathways, such as cytokine and cell-trafficking pathways (components of innate immunity), with the comparative lack of success of therapies that interfere with the more complex and flexible features of antigen-specific adaptive immunity.

Targets for immunotherapy

The treatment of human autoimmune diseases often occurs years after the onset of the pathogenic process, and despite our increasing knowledge of the cellular and molecular processes involved in immunity, the most effective targets for immunotherapy in the chronic phase of the disease are not obvious. Targeting various critical molecules involved in pathological pathways has led to the modulation of disease in animal models (Fig. 1). Components of the pathological cascade that have received most attention are: factors involved in lymphocyte homing to target tissues; enzymes that are critical for the penetration of blood vessels and the extracellular matrix by immune cells; cytokines that mediate pathology within the tissues; various cell types that mediate the damage at the site of the disease, as well as these cells' antigen-specific adaptive receptors, including the T-cell receptor (TCR) and immunoglobulin; and other toxic mediators, such as complement components and nitric oxide (Fig. 1).

A widespread misconception is that every step of the immune or pro-inflammatory process is a potential therapeutic target. Regrettably, this is not the case. Because most therapeutics only have a partial inhibitory effect, only those molecules that are in short supply (and thus rate-limiting) are likely to be useful targets. Therefore, therapy that specifically targets most of the steps (which are non-rate-limiting) in the immune or pro-inflammatory process yields little benefit in ongoing (late, active) autoimmune disease in humans. So far, only therapies that target two rate-limiting steps — the cytokine tumour-necrosis factor (TNF; ref. 5) and the molecule involved in lymphocyte homing, $\alpha_4\beta_1$ integrin[6] — have markedly ameliorated autoimmune disease progression; for example, in rheumatoid arthritis, inflamma-

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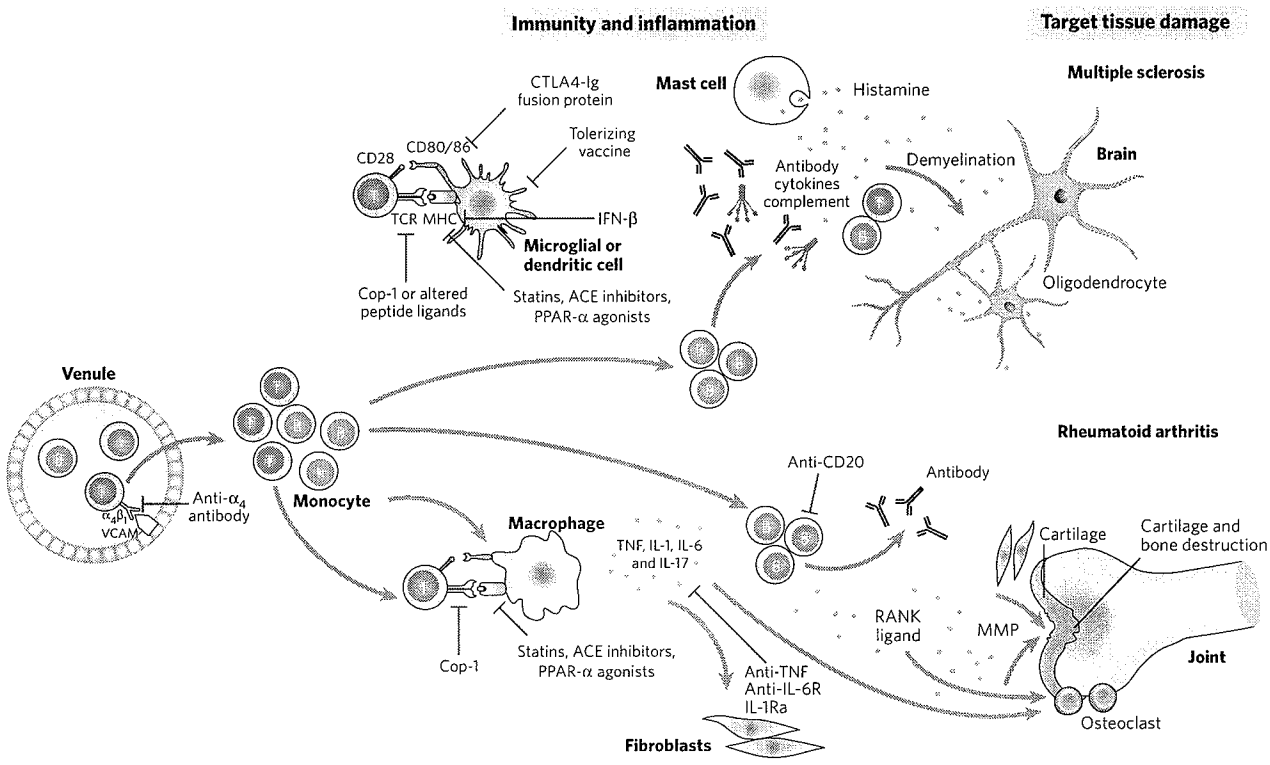

Figure 1 | Pathogenesis of multiple sclerosis and rheumatoid arthritis. Activated T cells express $\alpha_4\beta_1$-integrin, which binds to vascular cellular adhesion molecule (VCAM) on the surface of venules in inflamed tissues. This interaction allows the T cells to pass through the endothelial wall and penetrate the extracellular matrix. In multiple sclerosis (upper panel), the T cells re-encounter the cognate CNS antigen presented by MHC class II molecules on either microglial or dendritic cells. This interaction can be inhibited by glatiramer acetate (Cop-1) or altered peptide ligands. In addition, statins, angiotensin-converting enzyme (ACE) inhibitors, and PPAR-α agonists can all downregulate the inducible expression of MHC class II molecules. Similarly, cytokines such as interferon-β (IFN-β) downregulate MHC class II molecules and interfere with diapedesis of cells (the movement of cells through the endothelial wall) by downregulating metalloproteases. CD28 and CD80/86 interactions can be blocked by the CTLA4-Ig fusion protein. Tolerizing vaccines promote tolerance processes which occur when the T cell/dendritic cell interaction is not optimal. B cells and mast cells are also recruited into the inflammatory infiltrate. Antibody plus complement can produce 'membrane attack' complexes, which can damage the oligodendrocytes and underlying axon. Osteopontin is expressed on the surface of oligodendroglial cells and neurons during active disease, and is pivotal in the disease progression. In rheumatoid arthritis, T cells and macrophages that have entered the synovium from inflamed venules produce cytokines, especially TNF, IL-1, IL-6 and IL-17, which mediate damage to the synovium. This damage can be blocked by anti-TNF antibody, IL-1 receptor antagonist (IL-1Ra), and anti-IL-6 receptor (IL-6R) antibody. RANK ligand is the main signal for activating osteoclasts in cartilage, which mediate bone destruction. Anti-TNF antibodies reduce the migration of lymphocytes from the blood to the synovium, and also prevent bone loss by blocking the destructive effects of IL-1, IL-6 and TNF. Anti-CD20 kills B cells but not plasma cells; fibroblasts make most of the tissue-destructive metalloproteinases (MMPs).

tory bowel disease, ankylosing spondylitis, psoriasis and multiple sclerosis.

These particular key molecules and the processes they control can be referred to as 'tipping points'[7]. In epidemiology, a tipping point is defined as the moment when epidemics qualitatively change, reach a critical mass and have major repercussions. This concept is valuable in autoimmune diseases because many cellular and molecular processes contribute to tipping the balance towards the disease state, and therefore are potential therapeutic targets. But although targeting these tipping points may provide significant benefit, in terms of treating autoimmune disease, blocking these critical physiological molecules could also negate their beneficial roles in generating protective immune responses, and therefore could lead to an increased risk of infection. For example, despite the enormous success in treating multiple sclerosis by blocking $\alpha_4\beta_1$ integrin, this treatment was recently voluntarily withdrawn because of the development of a fatal untreatable infection. So tipping points are physiological processes that are key to maintaining both health and disease.

The targeting of TNF (ref. 8) or $\alpha_4\beta_1$ (refs 6, 9, 10) has remarkable effects on several autoimmune diseases, including rheumatoid arthritis, inflammatory bowel disease, ankylosing spondylitis, psoriasis and multiple sclerosis. These molecules can therefore be considered as true tipping points in the pathophysiology of autoimmune disease. But

Box 1 | Using commonly used drugs to treat autoimmunity

Recently, familiar oral medications, such as statins and angiotensin blockers, widely used for other disease conditions such as hypercholesterolaemia, hypertriglyceridaemia, allergy and hypertension, have been shown to inhibit some of the biochemical reactions that occur in autoimmune inflammation (Fig. 1). These drugs have shown promise in pre-clinical models of autoimmunity, as well as in early-stage clinical trials[11]. Even if they are not optimal therapies on their own, they are clearly pointing towards key alternative pathways, and may prove to be effective when used in synergy with other approaches.

Interestingly, the statins, which block the activity of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, and thus reduce levels of cholesterol, also inhibit the appearance of inducible MHC class II molecules[72]. The statins are remarkably potent in reversing disease in animal models, inducing shifts from the production of T_H1-type pro-inflammatory cytokines by autoaggressive T cells to T_H2-type cytokines[74]. Initial trials administering statins to multiple sclerosis and rheumatoid arthritis patients show moderate efficacy[71,75]. As with statins, peroxisome proliferator-activated receptor-α (PPAR-α) agonists — drugs used in type II diabetes — which regulate the activation of adipocytes and macrophages, also induce a shift in cytokine production from the T_H1 to T_H2 type[76]. Initial experiments suggest angiotensin blockers do the same[77]. The efficacy of the statins, PPAR-α agonists and angiotensin blockers may result from their ability to alter a number of pathological processes in the immune cascade.

because the benefit seen here is achieved by interfering with processes that are involved in both host defence and autoimmune pathology, the overall benefit:risk ratio is inherently difficult to predict.

In the pharmaceutical industry, drugs in only about 5% of the 'small-molecule' drug projects end up as approved therapeutics; most drop out because of problems, usually toxicity. Hence, existing drugs (which are relatively safe) with new potential uses present a wonderful opportunity. Recently, familiar oral medications, such as statins, which are widely used for other disease conditions, have been shown to be effective in animal models of autoimmunity and early-stage clinical trials in patients with multiple sclerosis and rheumatoid arthritis[11] (Box 1).

Non-antigen-specific approaches

T-cell populations and antigen-presenting cells

Despite preventing disease (such as arthritis and experimental autoimmune encephalomyelitis, EAE), to an impressive extent in animal models, anti-CD4-antibody therapy, with either lytic or non-lytic monoclonal antibodies, has not successfully treated human rheumatoid arthritis[12], psoriasis or multiple sclerosis[13]. However, the limited scope for experimentation in humans during clinical trials may mean that inappropriate antibodies or dose regimes have been used. Alternatively, failure to prevent disease might have been caused by the anti-CD4 antibody also inhibiting regulatory T cells that express CD4. By contrast, encouraging results have been reported from both animal models[14] and early clinical studies[15] using a mutated, less activating form of anti-CD3 antibody. The use of this antibody avoids the acute cytokine release — that causes a range of problems from malaise to hypotensive shock[16] — induced by non-mutated anti-CD3 antibody.

There is a growing consensus that antigen-presenting cells (APCs) are important rate-limiting cells for inducing immune responses[17]: a leading hypothesis is that inducible major histocompatibility complex (MHC) class II molecule expression is induced inappropriately on APCs at the site of autoimmune disease[18] (Fig. 1). Consistent with this, in many animal models of autoimmune disease, antibodies specific for MHC class II molecules reduce disease. But because the antibodies caused unexpected toxicity when tested in monkeys[19], this has not yet been tested in humans.

Effective antigen presentation and activation of T cells requires not only TCR recognition of MHC molecules complexed with a peptide, but also various ligand–receptor costimulatory interactions at the 'immune synapse' — the point of interaction between a T cell and an APC. Most important among these costimulatory interactions are CD28 molecules recognizing CD80 or CD86 molecules[11] (Fig. 2). Therapy using a cytotoxic T-lymphocyte antigen 4 (CTLA4)–immunoglobulin fusion protein, which blocks interactions with CD28, is effective in randomized, double-blind clinical trials in patients with psoriasis and rheumatoid arthritis[20], suggesting that even in late-stage disease, signals mediated by costimulatory molecules expressed by APCs are required. Blocking other molecules that are involved in activating the immune system may also be useful therapeutically. Unfortunately, despite promising results in experimental studies, the administration of an antibody specific for the T-cell-expressed costimulatory molecule CD40 ligand was toxic in humans, causing a number of deaths from thrombosis. The blocking of costimulatory molecules that are expressed only after antigen activation of T cells, such as OX40, may be efficacious and safer[21], as this would not block uninvolved T cells.

Regulatory T cells and B cells

Several regulatory subsets of T cells have been defined in recent years, and attention is now turning to their use for therapy. This is because defects in such regulatory subsets (in particular, the $CD4^+CD25^+$ regulatory T-cell subset) may be important in enabling autoimmune diseases to become established[22,23] (see review by Kronenberg and Rudensky in this issue, page 598).

Given the ubiquity of autoantibodies in autoimmune diseases, it was assumed that the antibody-producing cells — plasma cells and B cells

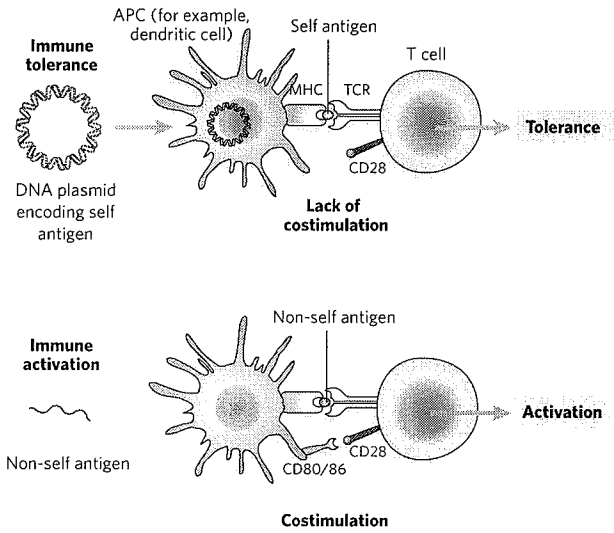

Figure 2 | Generating immune tolerance by using 'tolerizing' DNA vaccines. A DNA plasmid encoding a self antigen is transcribed and translated in a dendritic cell, but its expression does not stimulate the innate immune system enough to upregulate costimulatory molecules. A further reduction in costimulation is caused by the removal of CpG motifs in the plasmid. The presentation of self antigen by APCs without adequate costimulation leads to anergy or tolerance of T cells, because of the lack of interaction between CD28 with CD80 or CD86 (refs 72, 73). In contrast, conventional immunization, with a foreign antigen, leads to effective presentation of antigen in the MHC molecules with adequate costimulation, and leads to productive cytokine cascades and gene activation.

— would be a good target for therapy. However, this assumption has only recently been confirmed: lytic anti-CD20 antibody (rituximab; Rituxan), which lyses B cells, effectively treated rheumatoid arthritis and systemic lupus erythematosus[24], although extensive comedication of subjects in these trials makes the data difficult to interpret.

Cytokines

Cytokines are short-range protein mediators with a wide range of actions. They are important in all biological processes[25], including T-cell growth (IL (interleukin)-2, IL-4, IL-7, IL-15 and IL-21), inflammation (TNF, IL-1, IL-6 and IFN (interferon)-γ) as well as the inhibition of inflammation (IL-10, transforming growth factor-β (TGF-β) and IL-4). As extracellular molecules, they are accessible to 'biologicals' — protein therapeutics such as antibodies or soluble receptors.

The relative potency of cytokines that induce multiple biological effects is compatible with a rate-limiting, 'catalytic' role, and therefore they are potential therapeutic targets. A major problem in establishing which ones may be targets lies in the considerable overlap (redundancy) in their biological properties. Thus, IL-1, TNF, IL-6 and granulocyte–macrophage colony-stimulating factor (GM-CSF) have more than 80% overlap in function, when tested *in vitro*. So, which ones are likely to be therapeutic targets in which diseases? Insights into this problem have come both from *in vivo* experiments using animal models and from clinical studies[26].

In contrast to the limited success of treatment with cytokines (see below), blockade of cytokines is the success story of the current era of molecular therapy in autoimmunity, which is based on scientific analysis of disease mechanisms[8]. Research using joint tissue from patients with rheumatoid arthritis suggested the importance of TNF in the disease pathogenesis[27]. The existence of TNF-inhibiting biologicals (originally generated to treat sepsis syndrome) made it possible to perform a successful proof-of-principle clinical trial in 1992 (ref. 28) with the anti-TNF monoclonal antibody infliximab. This culminated

in the approval from 1998/1999 of a set of therapeutic biologicals: anti-TNF monoclonal antibodies (infliximab[29] and adalumimab[30]) and the TNF-receptor (TNFR) fusion protein (etanercept[31]; Enbrel). TNF blockade has demonstrated that biologicals can be used in the long term, and extensively: about a million patients have been treated with anti-TNF biologicals so far, and some for over seven years.

Much has been learnt from the use of anti-TNF biologicals; for example, the importance of finding the right therapeutic target. TNF is the body's fire alarm[5]. It initiates the defence response to local injury, recruits leukocytes, and initiates a whole series of events that are important in health and in many diseases. Hence its blockade is useful in treating many diseases. The mechanism-of-action studies (see Box 2) have provided several insights into the pathogenesis of targeted diseases[32], especially rheumatoid arthritis.

IL-6 is another useful target, with clinical-trial success in rheumatoid arthritis showing comparable efficacy to TNF blockade[33]. However, the clinical benefits of IL-6 blockade occur more slowly than with TNF blockade, as predicted from *in vitro* studies that revealed a TNF-dependent cytokine cascade[27,34], where TNF drives the production of multiple pro-inflammatory cytokines. Success has also come from IL-1 blockade using the IL-1-receptor antagonist anakinra, which is approved for the treatment of rheumatoid arthritis[35]. And promising results have been seen in the treatment of rheumatoid arthritis with anti-IL-15 antibody[36]. High mobility group 1 (HMGB1), a stimulator of inflammatory responses, is another promising target for arthritis and sepsis[37]. Finally, blocking the receptor activator of nuclear factor NF-κB ligand (RANKL), the main activator of osteoclasts, is a promising approach for reducing bone destruction, such as that seen in rheumatoid arthritis[38].

Cell recruitment: chemokines and adhesion molecules

The small-protein chemotactic cytokines (chemokines) have several properties that make them favoured targets in the pharmaceutical industry[39]: they are extracellular, and so accessible to biologicals; and they bind to seven-transmembrane receptors that can be blocked by small-molecular-mass chemicals. Most importantly, chemokines are mediators of cell migration. Because chronic inflammatory diseases depend on the recruitment of inflammatory cells to the inflamed site, any approach that reduces the number of inflammatory cells in the site of disease may be of benefit, be it through chemokine or adhesion-molecule blockade. However, like cytokines, there are numerous chemokines (more than 40) with redundant properties, so it is not clear which ones are the most relevant in which disease.

Immune surveillance is accomplished by highly mobile leukocytes that are primed to fight microbes anywhere in our bodies. Organ-specific autoimmunity may result when autoreactive lymphocytes enter an inflamed site, initiating multiple events[8,18]. Lymphocyte migration depends on highly specific 'adhesion' molecules expressed by T cells that bind to receptors induced on endothelial cells[40]. These adhesion molecules and their receptors have domains in the extracellular space, and so can be targeted with monoclonal antibodies. Because the key homing molecules — integrins and selectins — display a high degree of diversity, a particular integrin molecule or selectin molecule is critical for entry to a particular anatomical site, and blocking that molecule might abolish pathological homing to that site, leaving lymphocytes free to move elsewhere.

Initial studies in animal models of multiple sclerosis (EAE) indicated that the critical homing molecule to the inflamed central nervous system (CNS) is $\alpha_4\beta_1$ integrin[9]: anti-$\alpha_4\beta_1$ antibody blocked the entry of lymphocytes into the brain and abrogated the clinical paralysis associated with EAE. This approach also proved successful in patients with multiple sclerosis: a phase III trial of a humanized $\alpha_4\beta_1$-specific monoclonal antibody natalizumab (Tysabri) reduced clinical relapses by 66% over the next year, leading to Food and Drug Administration (FDA) approval of the drug[6]. Encouraging results were seen with the same antibody in the treatment of inflammatory bowel disease[10].

However, the blockade of $\alpha_4\beta_1$ integrin is not specific. It interferes with lymphocyte homing in general, and therefore raises the risk of opportunistic infections[11]. Recently, sales of natalizumab were withdrawn, after two patients taking it in combination with IFN-β1a (Avonex) developed progressive fatal multifocal leukoencephalopathy, an untreatable viral infection (http://www.fda.gov/cder/drug/infopage/natalizumab/default.htm). Blocking lymphocyte mobility with these two drugs, and blocking lymphocyte entry to the brain, may have caused this unusual infection, caused by the ubiquitous JC virus, the activation of which is most commonly seen in severely immunocompromised individuals.

Antigen-specific approaches

The adaptive autoimmune response becomes more complex as disease progresses, owing to the generation of T-cell reactivity and antibodies to other local molecules — a concept known as epitope spreading[41]. Thus, in the chronic stage of the disease, the adaptive immune response targets several different molecules at the anatomical site of the disease.

In the 1970s, a random copolymer of the amino acids glutamate, tyrosine, alanine and lysine (copolymer 1 or Cop-1), now termed glatiramer acetate or copaxone, was designed to mimic the composition of myelin basic protein (MBP) — a major target of autoimmune responses in multiple sclerosis. The administration of glatiramer acetate ameliorated EAE, and is now an approved drug for multiple sclerosis[42]: daily injection of glatiramer acetate reduces disease relapse by 30%, and induces a T helper 2 (T_H2)-type response to myelin antigens. This is desirable because T_H1-type responses to myelin proteins are pathogenic. However, T_H2-type responses are associated with allergic reactions, and about 10% of individuals taking glatiramer acetate develop allergic reactions.

An altered peptide ligand (APL) of MBP-derived peptide 83–99 was constructed by mutating the amino acids that form the main contact sites with the TCR on disease-causing T cells[43]. The administration of the

Box 2 | Anti-TNF therapy of rheumatoid arthritis

Mechanism of action

- Reduction in pro-inflammatory cytokine cascade, including reduction of IL-6, IL-1, GM-CSF and vascular endothelial growth factor (VEGF).
- Reduction in leukocyte trafficking owing to decreased expression of adhesion molecules and chemokines.
- Reduction in tissue-destructive enzymes, such as matrix metalloproteinases (MMPs), but levels of tissue inhibitor of MMPs are maintained.
- Reduction in angiogenesis through reduced VEGF production.
- Normalization of abnormal haematology: haemoglobin restored, platelets and fibrinogen reduced.

Clinical benefits

- Reduction of symptoms including pain, stiffness and lethargy.
- Reduction in signs of active disease including tenderness and joint swelling.
- Reduction in cartilage and bone damage.
- Induction of tissue repair.

Potential side effects

- Increased risk of infection due to reduced cytokine, for example increased risk of TB and pneumonia.
- Increased levels of antibodies to double-stranded DNA; rare cases of drug-induced lupus can occur.
- Increased risk of lymphomas (not proven).

Differences between TNF-blocking drugs

- Etanercept blocks TNF and lymphotoxin a (LTa).
- Infliximab and adalumimab, but not etanercept, are active in Crohn's disease.
- Difference most likely to be due to different dosing regimes.
- Alleged differences in cytotoxicity/apoptosis are controversial.

Table 1 | Therapeutics for human autoimmunity

Target/therapeutic	Status of therapeutic	Disease outcome	Disadvantages	References
Cytokines				
TNF-specific monoclonal antibody	Approved for rheumatoid arthritis, Crohn's disease, psoriatic arthritis and ankylosing spondylitis	Improvement in disability in all diseases; joint repair in rheumatoid arthritis	Increased risk of TB and other infections; slight increased risk of lymphoma	28–30, 32
Soluble TNFR fusion protein	Approved for rheumatoid arthritis, psoriasis and ankylosing spondylitis	Clinical benefit is the same as TNF-specific monoclonal antibody	Risks are the same as TNF-specific monoclonal antibody therapy	31, 32
IL-1-receptor antagonist	Approved for rheumatoid arthritis	Improves disability	Relatively low efficacy Daily injection	34, 35
IL-15-specific monoclonal antibody	Phase II trial for rheumatoid arthritis	Promising results for disability	Potential for opportunistic infection (blocks natural killer (NK) cells, CD8 memory)	36
IL-6-receptor-specific monoclonal antibody	Phase II trial for rheumatoid arthritis	Decreased disease activity	Potential for opportunistic infection	71
Recombinant type 1 interferons	Approved for relapsing/remitting multiple sclerosis	Reduction in relapse rate	Liver toxicity; influenza-virus like syndrome is common	59
Integrins				
$\alpha_4\beta_1$-integrin-specific monoclonal antibody	Approved for relapsing/remitting multiple sclerosis Phase II/III trials for rheumatoid arthritisand inflammatory bowel disease	Reduction in relapse rate; delay in progression of disability at two years; encephalopathy	Increased risk of infection Progressive multifocal encephalopathy	6, 10
Oral small-molecule inhibitors	Phase I trials in progress	Not yet known		
HMG-coenzyme A reductase				
Statins	Phase II trials for multiple sclerosis	Reduced activity on magnetic resonance scans	Hepatotoxicity, rhabdomyolysis	72
T cells				
CD3-specific monoclonal antibody	Phase II trials for type 1 diabetes	Reduced insulin usage	Increased risk of infection	14–16
CTLA4-immunoglobulin recombinant protein	Phase III trials for rheumatoid arthritis, psoriasis and multiple sclerosis	Improvement in rheumatoid arthritis		20
B cells				
CD20-specific monoclonal antibody	Phase II trials for rheumatoid arthritis, systemic lupus erythematosus (SLE) and multiple sclerosis	Improvement in rheumatoid arthritis SLE (although extensive co-medication makes interpretation problematic)	Possible increased risk of infection especially if re-treated	24
Antigen-specific T-cell responses				
Random copolymer glatiramer acetate	Approved for relapsing/remitting multiple sclerosis	Reduction in relapse rate	Allergic reactions in 10% of patients	44, 45
Altered peptide ligand to MBP peptide 83-99	Phase IIb trials for multiple sclerosis	Reduced brain lesions (at low doses)	Can exacerbate disease at high doses	46
Altered peptide ligand to HSP60 peptide	Phase II trials for type 1 diabetes	Reduced insulin usage	Allergic reactions in 10% of patients	11
Altered peptide ligand to insulin peptide	Phase II trial in progress for type 1 diabetes	Not yet known	Not yet known	
MBP-encoding tolerizing DNA vaccine	Phase I/II trial in progress for relapsing/remitting multiple sclerosis	Not yet known	Not yet known	11, 48

MBP APL ameliorated EAE in mice induced by a different myelin protein (proteolipid protein), even when the APL was administered after the initial attack of paralysis[43]. And APL administration similarly induced a shift to T_H2-cytokine production, reduced epitope spreading, and reduced the broadening of the adaptive T- and B-cell responses. In a phase II placebo-controlled human clinical trial, MBP APL (given in weekly subcutaneous doses) shifted the response of MBP-specific T cells, promoting T_H2-cytokine production (including IL-4, IL-5, IL-10 and IL-13) and downregulating T_H1-cytokine production (including IFN-γ and TNF)[44]. Lower doses of MBP APL reduced both the number and the volume of brain lesions detected with magnetic resonance imaging (MRI), but higher doses exacerbated disease in three patients and increased brain lesions[45]. A phase IIb trial is now underway using the lower dose. Three other trials of antigen-specific therapy are underway or recently completed for type 1 diabetes mellitus (T1DM), including phase II trials with glutamic acid decarboxylase, and trials with APLs of an insulin peptide or of a heat-shock protein 60 (HSP60) peptide. In the trial with the APL of HSP60, decreased exogenous insulin use was observed in diabetics, as well as a T_H2 shift[46,47].

An alternative method of targeting antigen-specific responses has recently been developed using DNA constructs that are designed to promote the tolerization of immune responses to multiple myelin components. These DNA constructs encode several myelin antigens, where immune stimulatory motifs (CpG motifs) in the DNA, which promote expression of costimulatory molecules (such as CD28), are replaced with immunosuppressive motifs (GpG motifs), leading to sub-optimal costimulation of antigen-specific T cells (Fig. 2; ref. 48). When administered to mice after the first signs of EAE, these DNA plasmids reduced the subsequent relapse rate over the next three months by more than 50%, and also reduced the spreading of autoantibody responses. A phase I trial with DNA vaccines designed to tolerize against myelin proteins is currently underway.

Oral administration of myelin antigens in multiple sclerosis, collagen in rheumatoid arthritis and insulin in T1DM (which has been shown to favour tolerization of immune responses) has been tested. Despite successfully preventing disease in animal models when antigen was fed at the time of disease induction[49,50], clinical trials attempting to treat ongoing disease have been unsuccessful[51]. A summary of therapeutics is in Table 1.

Current tools for immunotherapy

Monoclonal antibodies

The success of monoclonal antibodies was slow to arrive, but in 2004, there were two 'blockbusters' on the market (each generating over $1 billion) — infliximab (Remicade), an anti-TNF antibody, and rituximab, an anti-CD20 antibody. More are on the way; currently almost half of all drug candidates in clinical development are monoclonal antibodies. Infliximab and rituximab are derived from early monoclonal antibody technology. They are 'chimaeric' antibodies, consisting of a mouse combining site (Fv) while the rest (about 70%) is human[52]. Subsequent developments have led to 'humanized' antibodies, in which mouse-derived variable regions (or complementarity-determining regions, CDRs) are grafted into a human antibody scaffold, and 'fully human' antibodies, which contain human variable-region components selected by phage display[53]. Humanized monoclonal antibodies in the clinic include natalizumab, which blocks $\alpha_4\beta_1$ (ref. 6), and the fully human anti-TNF antibody adalimumab[30] (Humira).

Because many potential therapeutic targets are exposed in extracellular fluids (cytokines, chemokines, receptors, other cell-surface molecules and adhesion molecules), they are readily accessible to high-affinity neutralizing antibodies. Furthermore, as natural-body constituents (in contrast to the small-molecule chemicals commonly used as pharmaceuticals), antibodies intrinsically lack toxicity when manufactured, purified and handled properly. Therefore, any toxicity that does occur with monoclonal antibodies is likely to be mechanism related. Another benefit of monoclonal antibodies lies in the fact that even partially humanized antibodies (such as chimaeric antibodies of mouse Fv on a human backbone), as well as fully humanized antibodies, are relatively non-immunogenic. This is probably due to the phenomenon of 'high zone tolerance' described in the 1960s and 1970s that occurs with deaggregated human immunoglobulins[54] (whereby intravenous deaggregated gammaglobulin was tolerogenic if given in high doses) and the concomitant use of methotrexate, which has immunosuppressive as well as autoinflammatory effects[29].

Receptor fusion proteins

Receptor fusion proteins are proteins in which the binding site of a receptor is fused onto an antibody Fc region, which improves the protein's half-life and other pharmacological properties. The most successful receptor fusion protein is etanercept, a dimeric p75 TNFR–immunoglobulin G (IgG) Fc fusion protein[31] (with sales of over $1 billion). The clinical benefit of etanercept is indistinguishable from that of anti-TNF antibodies in rheumatoid arthritis[32,55], psoriasis and ankylosing spondylitis, although anti-TNF antibodies are more effective in the treatment of inflammatory bowel disease. Receptor fusion proteins are more expensive to manufacture than antibodies, and the use of natural receptors provides for less diversity than with antibodies.

Cytokines

Cytokines have some useful 'drug-like' properties, such as potency, but also some disadvantages, such as a short half-life. But the main problem with cytokines is that they have multiple effects on many cell types[25], so systemic injection of cytokines can cause undesirable effects. Thus, the efficacy in animal models of the endogenous anti-inflammatory cytokines IL-10 (ref. 56), IL-4, IL-11 and TGF-β has not translated into their use as human therapeutics, owing to their toxicity. However, the local regulated delivery of cytokines using gene therapy could make them effective as treatments. Recently, it has become possible to engineer cytokines that have enhanced half-lives and are activated only at a desired location[57,58]. Such modifications may overcome some of the inherent difficulties of cytokine therapy.

The type 1 interferons, IFN-α and IFN-β, are effective drugs and have been approved for use in viral infections, some cancers and multiple sclerosis. In multiple sclerosis, relapse rates are reduced by 30% with the administration of IFN-β (ref. 59). However, flu-like symptoms are common during therapy with IFNs, and the immunogenicity of IFNs (probably mechanism related because they upregulate antigen presentation) can limit their efficacy. IFN-β inhibits the activity of metalloproteases 2 and 9. This protease activity is required for lymphocyte homing, so when the administration of IFN-β is combined with adhesion cell blockade, lymphocyte entry into an organ may be drastically reduced[11]. In this circumstance, endogenous viruses like JC virus, which causes progressive multifocal leukoencephalopathy, may become activated with fatal consequences.

Mutated versions of cytokines can be used as decoys, inhibiting the ability of the endogenous cytokine to act on its receptor. This has been reported with TNF variants that bind to non-mutated endogenous TNF, with the resulting trimeric complex unable to activate TNFRs. In animal models, these TNF variants are effective[60].

Box 3 | Combination drug therapy in serious diseases

Because we do not know the cause of chronic autoimmune diseases, it is unlikely that any single therapy can halt or reverse all the troubling manifestations of these diseases. The way that candidate therapies are often tested — in isolation — predisposes such therapies to failure: in isolation, their effect on a highly complex multifactorial disease process may be relatively small.

Clinically, there has been marked success in the treatment of rheumatoid arthritis by combining methotrexate — an anti-proliferative folic acid inhibitor that inhibits T cells (and other cells) — with TNF inhibitory drugs[29]. This has been followed by combining methotrexate with other therapeutics, including anti-IL-6R (ref. 33) antibody and CTLA4-immunoglobulin fusion protein[20]. Methotrexate in combination with anti-TNF therapy was used in an attempt to mimic the augmented benefit of anti-CD4 and anti-TNF antibodies[79]. The lesson here, as in cancer therapeutics, is that more clinical efficacy (and less toxicity) may result from partially blocking several pathways than from complete blockade of any one pathway, which in humans is unattainable. However, certain combinations may be risky. For example, blocking TNF and IL-1 augments the risk of infection[80] and so caution is necessary to avoid diminishing the benefit:risk ratio.

It is likely that as we understand more about the rate-limiting steps or the 'tipping points' in disease processes, better combinations will be devised to maximise efficacy and to minimize side-effects, the duration of treatment and its cost.

Overcoming limitations

Although there is a lot of optimism among some circles that many new safe therapies are just around the corner, this hope belies the fact that clinical successes, where the benefits outweigh the risks, are few and far between. The failures include antibodies specific for cell-surface antigens such as CD4 and CD25, cytokines such as IL-8, fusion proteins such as the IL-1-receptor 'trap' and the TNFRp55–immunoglobulin fusion protein lenercept, and multiple antigen-specific approaches.

It is thus comforting that there are some clear successes, such as TNF blockade, that are now well established (Table 1). However, the recent withdrawal of anti-$\alpha_4\beta_1$ integrin emphasizes the complexity of reversing ongoing autoimmune disease, without provoking serious complications. Understanding the risk versus benefit relation requires more time than is usually spent in pre-clinical models, and often takes thousands of patient-years of experience to be established.

As summarized in Box 2, anti-TNF therapy of rheumatoid arthritis has marked clinical benefit, with some changes, such as reduction in tiredness, occurring within hours. This benefit occurs in most patients whose condition has not improved following other treatments, such as methotrexate. However, the degree of clinical benefit can vary considerably from patient to patient. The greatest benefit is

seen with combination therapy (Box 3). On the basis of single parameters only, such as joint swelling, all patients improve to some degree[28], but if compound parameters are monitored, such as the American College of Rheumatology (ACR) criteria (including number of swollen and tender joints, and levels of C-reactive protein), response rates vary between 50–60% in late-stage disease[29], to more than 80% in the early-stage disease[61]. In the early stage of disease, there is evidence of disease remissions, which may persist for a year or more after the withdrawal of anti-TNF therapy[61] (F. Breedveld *et al.*, unpublished observations). So early treatment may be the most beneficial and cost-effective. But there is, as yet, no evidence of a cure.

Anti-TNF therapy reduces joint pathology, even in patients showing no clear benefit according to ACR criteria. This suggests that the links between inflammation and joint damage are not fully understood. Most importantly, recent studies have documented evidence for joint repair, after TNF blockade: joint X-rays taken after one year of treatment show an improvement in joint condition compared with those taken before treatment[62,63]. It is the first example of therapy promoting endogenous repair in any reported human disease.

The most predictable problem of therapy with TNF blockade (and most other immunotherapies including anti-$\alpha_4\beta_1$-integrin antibody) is augmentation of the risk of infection. In this case, the magnitude of this risk is hard to measure because rheumatoid arthritis patients are more susceptible to infections, partly owing to the disease and partly because of other treatments. The initial incidence of tuberculosis (TB)[64] in one in every 2,000 patients treated with anti-TNF has been reduced markedly by screening and, if necessary, administration of prophylactic therapy. Other opportunistic infections are rarer, but like TB can occasionally be lethal. More common are respiratory infections. The consensus at present is that the benefit of using TNF blockade in autoimmune diseases with a bad prognosis outweighs the risks[65,66].

The risk of infection could be reduced if the duration of TNF blockade were briefer; for example, by using small-molecule chemicals of short half-life. The dilemma here, however, is to define the right therapeutic target. Attempts so far to develop inhibitors of p38 MAP kinase — a component of pro-inflammatory signalling cascades and a favourite target among pharmaceutical companies — have not succeeded, owing to toxicity. Other interesting small-molecule targets, such as IKK2 (inhibitor of NF-κB kinase 2), are also risky choices because of their presence in almost all cells. Another approach is to target the mechanism involved in the production of TNF in the joints versus that involved in the production of TNF in the immune system, but despite evidence that the mechanism differs, we do not know the molecular targets[67].

Another common side effect of TNF blockade is the induction of IgM anti-nuclear antibodies, which have been detected in many patients (15%; ref. 68), although IgG antibodies and drug-induced lupus (an antibody-mediated disease) only rarely occur (less than one in 1,000 patients). If lupus does occur, it is reversible, treatable and not nephrotoxic, so is not a major clinical problem.

Lymphomas are more frequent in patients with rheumatoid arthritis than in the normal population, especially those with severe long-standing disease. However, as severe disease is treated by anti-TNF therapy, it is not yet clear whether there is an increased risk of developing lymphomas[69] after anti-TNF therapy.

Benefit from anti-TNF blockade is not seen in all autoimmune diseases. In fact, the treatment of multiple sclerosis patients with TNF blockade, using lenercept, a TNFRp55–Fc construct which never reached the market, exacerbates the frequency of disease relapse[8], possibly by augmenting T-cell activity[70]. This discordance may be explained, in part, by the inability of the TNFR fusion constructs to penetrate the inflamed brain owing to the endothelial blood–brain barrier. Alternatively, although TNF may have a destructive role in inflammation in the brain, it may also act as a growth factor for myelin-producing cells, indicating that TNF, similar to many other cytokines, has both harmful and beneficial effects[11].

Outlook

Two decades of work defining the molecular basis of the immune response is starting to pay off in the field of autoimmunity. A whole set of 'targeted therapies' has been developed to block many steps in the immune and pro-inflammatory response. Of these, several successes have had a profound impact on patients, on our understanding of disease mechanisms and even on the pharmaceutical industry. The variety of potential therapeutic targets is enormous, but we do not know the rules that define targets of various quality, in terms of their efficacy as well as safety. Some molecules and pathways are common in many autoimmune diseases. TNF is the best-documented example. Hence, TNF blockade is an approved therapy for multiple chronic inflammatory diseases — rheumatoid arthritis, Crohn's disease, psoriatic arthritis, ankylosing spondylitis and psoriasis, with more likely to follow.

The future goal will be to improve the efficacy of immunotherapy, from the current state of partial disease control, to increased disease control, to establishing remission and eventually to cure, without increasing either the risks or costs of treatment. An important step in this progression will be achieving earlier treatment.

For now, the non-antigen-specific approaches are the ones yielding clinical benefit, with the blocking of cytokines, and possibly adhesion molecules, being the most effective. But with such non-antigen-specific approaches, the risk of opportunistic infection is problematic. In the future, non-antigen-specific approaches may be made safer by targeting them to the site of disease, for example by gene therapy. But the most obvious way to reduce opportunistic infections is to use antigen-specific therapy — a dream of immunologists for generations now. Although several attempts in the past decade have failed, we are optimistic that eventually, the molecular understanding of tolerance and immunity will progress, and the 'holy grail' of autoimmunity — long-term antigen-specific therapy — will be reached. The progress made in devising rational and effective non-antigen-specific therapy reflects the development of useful research and therapeutic tools, and provides grounds for this optimism.

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Competing interests statement The authors declare competing financial interests: details accompany the paper on **www.nature.com/nature**

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PNAS 2000;97;6681-6686
doi:10.1073/pnas.97.12.6681

A critical role for eotaxin in experimental oral antigen-induced eosinophilic gastrointestinal allergy

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Edited by K. Frank Austen, Harvard Medical School, Boston, MA, and approved April 11, 2000 (received for review March 6, 2000)

Despite marked advances in the understanding of allergic responses, the mechanisms regulating gastrointestinal allergy are not very well understood. We have developed a model of antigen-induced eosinophil-associated gastrointestinal allergy and characterized the role of eotaxin and IL-5. Challenge of allergen-sensitized mice with oral allergen, in the form of enteric-coated beads, resulted in marked allergen-specific IgG_1 and IgE, Th_2-type (IL-4 and IL-5) cytokine production, and eosinophil accumulation in the blood and small intestine. In the genetic absence of eotaxin, a chemokine constitutively expressed in the gastrointestinal tract, eosinophil recruitment into the small intestine was ablated, and these mice developed enhanced eosinophil accumulation in the blood compared with wild-type mice. Interestingly, in the absence of IL-5, allergen challenge promoted partial eosinophil accumulation into the small intestine and a decline in circulating eosinophil levels. Collectively, these results establish that the accumulation of gastrointestinal eosinophils is antigen induced, can occur independent of IL-5, and provides a molecular mechanism to explain the dichotomy between peripheral blood and tissue eosinophilia. Furthermore, eotaxin is identified as a critical regulator of antigen-induced eosinophilic inflammation in the gastrointestinal tract.

Allergic diseases have reached epidemic proportions in the Western world, affecting nearly 30 percent of the population (1). Interestingly, this increased prevalence is paralleled by an increase in the severity and spectrum of disorders involving hypersensitivity responses in various tissues (e.g., the gastrointestinal tract). Although substantial progress has been made in elucidating the inflammatory mechanisms involved in allergic responses in the lung, there has been limited progress in understanding the pathogenesis of allergic disorders of the gastrointestinal tract. The development of experimental models of allergy has provided important insights into the immunological mechanisms regulating systemic (e.g., anaphylaxis) and pulmonary (e.g., asthma) allergic diseases. Collectively, these studies have identified a central role for cytokines (e.g., IL-4, IL-5, and IL-13), $CD4^+$ T cells, mast cells, and, in particular, eosinophils, in the induction and sustainment of allergic inflammatory responses (2).

Eosinophil accumulation in the peripheral blood and tissues is a hallmark of allergic responses, and clinical investigations have established a strong link between the pathobiology of several allergic disorders and eosinophil accumulation and activation (3). However, it remains to be determined why some disease states are characterized by peripheral blood eosinophilia, whereas others are associated with a tissue eosinophilia in the presence or absence of peripheral blood eosinophilia. Although most studies have demonstrated an integral role for the cytokine IL-5 in eosinophil trafficking during allergic inflammatory responses, chemokines have also been recently implicated in the regulation of eosinophil accumulation (4–6). In particular, eotaxin has been identified as a potent and selective eosinophil chemoattractant and has been implicated in the pathogenesis of human allergic disease (7). However, in contrast to mice deficient in IL-5, aeroallergen challenge of eotaxin-deficient mice induces eosinophilic airway inflammation (8–10). Collectively, these data suggest that in comparison to eotaxin and other CCR3-ligands, IL-5 plays an obligatory role in regulating eosinophil trafficking during allergic responses in the lung.

There are a spectrum of eosinophil-associated inflammatory responses in the gastrointestinal tract, including IgE-mediated food anaphylaxis, inflammatory bowel disease, gastroesophageal reflux, allergic eosinophilic gastroenteritis, and eosinophilic colitis (11). It is currently thought that eosinophils may augment and sustain the gastrointestinal inflammatory response through the release of inflammatory mediators and/or granule cationic proteins that are toxic to the mucosa (11–14). However, although there have been recent advances in modeling some of these disease processes (e.g., IgE-mediated anaphylaxis responses), there have been only limited models of eosinophil-associated gastrointestinal allergy, and the precise mechanisms regulating gastrointestinal eosinophilia and the immunopathological role of this leukocyte in gastrointestinal disorders remain an enigma (11, 13, 15). To elucidate these processes, we have developed a murine model of eosinophil-associated gastrointestinal allergy and examined the role of eotaxin and IL-5 in the regulation of eosinophil trafficking.

Methods

Animals. Eotaxin-deficient inbred mice of the (129/SvEv) strain were maintained with age, and sex-matched controls were obtained from Taconic Farms as previously described (9). IL-5-deficient inbred mice of the (BALB/c) strain and age and sex-matched controls were kindly provided by K. Matthaei (John Curtin School of Medical Research, Canberra, Australia) (8). Mice were sensitized by i.p. injection with 50 μg ovalbumin (OVA)/1 mg alum in 0.9% sterile saline on day 0. On days 12 and 15, mice were lightly anesthetized with Metofane inhalation (methoxy-fluorane; Pittman–Moore, Mundelein, IL) and orally administered 20 mg of encapsulated OVA enteric-coated beads or encapsulated placebo enteric-coated beads followed by oral administration of 300 μl of acidified H_20 (pH 2.0) (16). Seventy-two hours after the last antigen challenge, mice were killed by cervical dislocation and parameters measured.

ELISA Measurements. Serum OVA-specific IgG_1 and IgE concentrations were determined by ELISA. Sample wells were coated with OVA (100 μg/ml) for IgG_1 or anti-mouse IgE (EM-95; 10 μg/ml; gift from F. Finkelman, University of Cincinnati, Cincinnati, OH), blocked with 10% FBS in PBS, and washed with 0.05% Tween-20 in PBS. Serum samples were diluted 1:5 for IgE and 1:1,000 for IgG_1 with 10% FCS in PBS and serially diluted (1:2). After a 2-h incubation at 37°C, plates were washed with 0.05% Tween-20 in PBS, and biotin-conjugated anti-mouse IgG_1 (clone: A85–1; 0.5 μg/ml; PharMingen) or biotinylated OVA (4 μg/ml) was added. By

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: MBP, major basic protein; OVA, ovalbumin.

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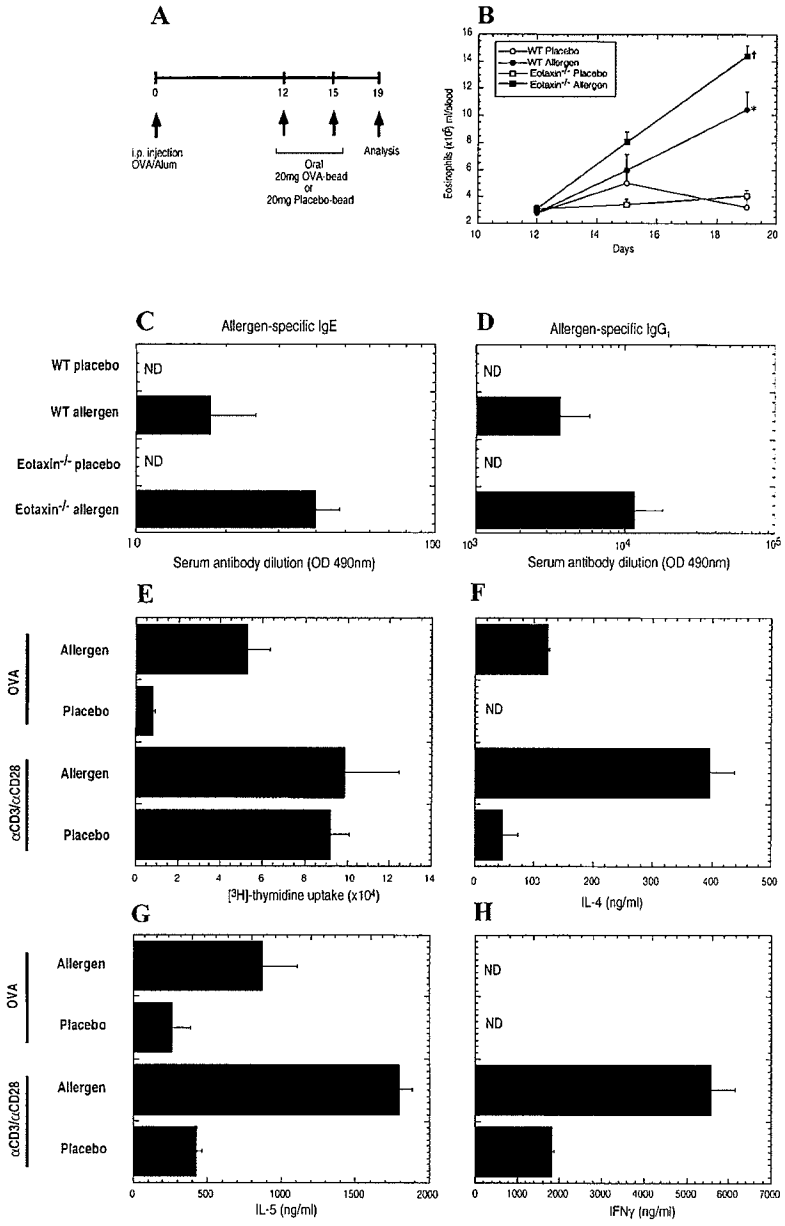

Fig. 1. Characterization of gastrointestinal allergy in wild-type and eotaxin-deficient mice. (*A*) Experimental regime. Mice were i.p. injected with 50 μg OVA/1 mg alum on day 0. On days 12 and 15, mice were orally administered 20 mg of encapsulated placebo- and OVA-enteric coated beads followed by acidified H_2O (pH 2.0). Four days after the last challenge, mice were killed and parameters measured. (*B*) Eosinophil numbers in the peripheral blood of placebo- and oral-allergen-challenged wild-type (WT) and eotaxin-deficient (eotaxin$^{-/-}$) mice. *Inset* specifies the groups. (*C* and *D*) Levels of allergen-specific IgE (*C*) and IgG_1 (*D*) in the serum of placebo- and oral allergen-challenged wild-type (WT) and eotaxin-deficient (eotaxin$^{-/-}$) mice were determined by ELISA. (*E*) Proliferation of splenic T cells stimulated by αCD3/αCD28 or OVA were measured by [^{3}H]-thymidine incorporation. (*F–H*) Secretion of IL-4 (*F*), IL-5 (*G*), and IFNγ (*H*) by αCD3/αCD28- and OVA-stimulated splenic T cells. Data in *B* and *D* represent the mean ± SEM for groups of four to five animals in triplicate experiments and (*E–H*) mean ± SEM from three individual cultures obtained from four to five mice in each group. Statistical significance of experimental groups was analyzed by using the unpaired Student's *t* test (*B*). *, $P < 0.05$ compared with day 19 allergen challenge of wild-type mice; +, $P < 0.001$ compared with placebo challenge of wild-type mice on day 19. Unless otherwise indicated, data were obtained on day 19. ND, not detected.

using streptavidin horseradish peroxidase detection (2 μg/ml; ImmunoTech, Marseilles, France), the OD was read at 490 nm within 30 min. Data represent mean ± SEM of the serum dilution required to obtain an OD = 0.4. Values are representative of n = 4–5 mice per group from triplicate experiments.

Antigen-Specific T-Cell Response. Splenocytes were subjected to OVA or αCD3/αCD28 stimulation as previously described (17). For proliferation experiments, splenocyte cultures were pulsed with 1 μCi [^{3}H]-thymidine for the last 6 h of a 72-h culture. IL-4, IL-5, and IFNγ levels were determined in the supernatants from OVA- (50 μg/ml) or αCD3- (5 μg/ml)/αCD28 (1 μg/ml) stimulated splenocytes homogenates by using the OptEIA Mouse IL-4 and IL-5 kits (PharMingen), respectively. The IFNγ level was measured by using rat anti-mouse IFNγ mAb (PharMingen; 5 μg/ml, clone; R4–6A2). The sensitivity of the ELISA system was 20 pg/ml for IL-4 and IL-5 and 100 pg/ml for IFNγ.

Immunohistochemistry. The small intestine of mice was divided into the duodenum, jejunum, and ileum, and segments of the gastrointestinal tract were fixed with paraformaldehyde, processed by using standard histological techniques, and immunostained with antiserum against mouse major basic protein (MBP) as previously described (18). Briefly, 5-μm sections were quenched with H_2O_2, blocked with normal goat serum, and stained with a rabbit antimurine eosinophil MBP antiserum (gift of J. and N. Lee, Mayo Clinic, Scottsdale, AZ). The slides were washed and incubated with biotinylated goat anti-rabbit antibody and avidin–peroxidase complex (Vectastain ABC Peroxidase Elite kit; Vector Laboratories). The slides were then developed by nickel diaminobenzidine, enhanced cobalt chloride to form a black precipitate, and counterstained with nuclear fast red. Quantification of immunoreactive cells was performed by morphometric analysis by using the Metamorph Imaging System (Universal Imaging, West Chester, PA). The sections were taken

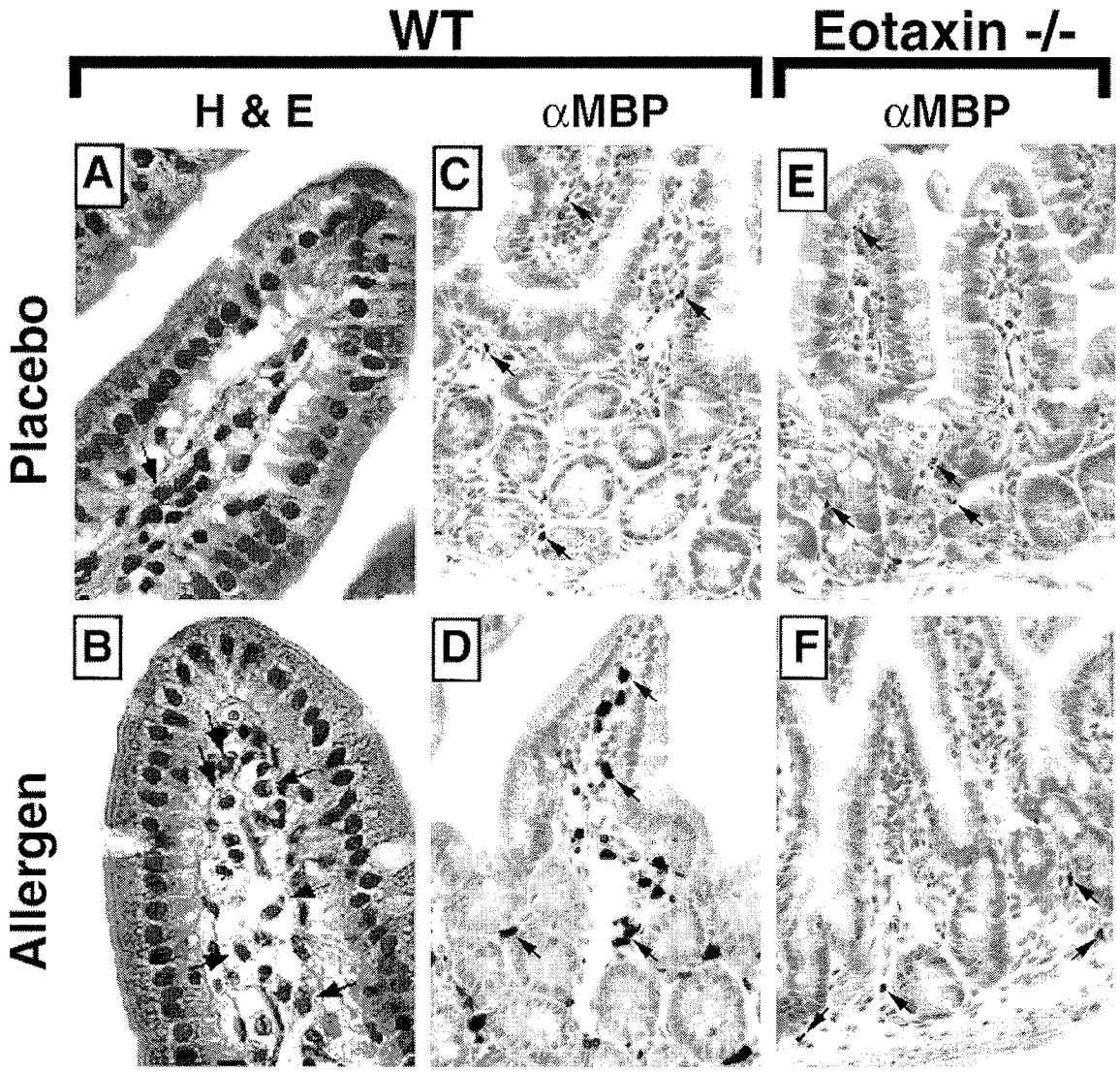

Fig. 2. Histological analysis of jejunum tissue from placebo- and oral allergen-challenged wild-type and eotaxin-deficient mice. (*A* and *B*) Photomicrographs represent hemataoxylin- and eosin-stained jejunum sections from placebo- (*A*) and oral allergen- (*B*) challenged wild-type mice. (*C* and *D*) Photomicrographs represent rabbit anti-mouse MBP [αMBP]-immunostained jejunum sections from placebo- (*C*) and oral allergen- (*D*) challenged wild-type mice. (*E* and *F*) Photomicrographs represent anti-MBP-stained jejunum sections from placebo- (*E*) and oral allergen- (*F*) challenged eotaxin-deficient mice. In placebo-challenged wild-type mice (*C*), eosinophils are predominantly localized to the crypt region and less frequently within the lamina propria of the villus. In oral allergen-challenged wild-type mice (*B* and *D*) but not eotaxin-deficient mice (F), an eosinophilic infiltrate is observed. Eosinophils are present within the mucosa and throughout the length of the villus. Arrows depict representative eosinophils. (*A* and *B*, ×950; *C–F*, ×460.)

from the same position in the jejunum (3–5 cm distal to the stomach), and at least four to five random sections per mouse were analyzed. Values were determined by quantifying the total MBP^+ pixel number relative to the total pixel counts of the lamina propria and mucosa regions of the gastrointestinal tissue. Eosinophil levels are expressed as the MBP staining/area (%).

FACS Analysis on Peripheral Blood Eosinophils. Peripheral blood was subjected to red blood cell lysis, and cells (10^6) were incubated with 1 μg phycoerythrin-conjugated anti-mouse LPAM-1 (Integrin $\alpha_4\beta_7$ complex; DATK32; PharMingen) and FITC-conjugated anti-mouse CCR3 mAb (1/200; DNAX) or the isotype-matched control antibodies at 4°C (19). Cells were analyzed on a FACScan Flow cytometer (Becton Dickinson) by using CELLQUEST software (Becton Dickinson).

Results

Development of Experimental Antigen-Induced Eosinophilic Gastrointestinal Allergy. One of the complexities of inducing allergic inflammation of the gastrointestinal tract is the ineffectiveness of orally administered soluble protein antigens in promoting hypersensitivity responses; rather, oral antigens generally promote immunological tolerance (20). The poor immune response and induction of oral tolerance is thought to be associated, at least in part, with gastric digestion of soluble protein antigens, which leads to the formation of nonimmunogenic peptides (21, 22). To overcome immunological tolerance associated with oral administration of soluble antigens (23, 24), we used a system whereby a soluble protein antigen was encapsulated to protect against gastric digestion (16). The encapsulated biodegradable antigen particles are resistant to degradation at gastric pH (pH 2.5); however, they are susceptible

to degradation at pH 5.5, which facilitates the delivery and release of the allergen in a preserved native conformational state to the small intestine (16). Extensive previous investigations have demonstrated that the particles may overcome immunological tolerance associated with oral administration of antigens and possess adjuvant and immunostimulatory activity promoting antigen-specific antibody production (25).

Mice were injected i.p. with the egg antigen OVA in the presence of adjuvant (alum) and subsequently challenged with oral OVA particles on days 12 and 15 (Fig. 1*A*). Administration of oral allergen to sensitized mice induced peripheral blood eosinophilia and allergen-specific IgE and IgG_1, but not IgG_{2a}, antibody responses (Fig. 1 *B–D*; data not shown). On day 19, the level of eosinophils in the peripheral blood of oral allergen-challenged mice was 3.5-fold higher than in placebo-challenged mice (Fig. 1*B*). To establish whether the peripheral blood eosinophilia and antigen-specific IgE was associated with the development of a $CD4^+$ Th_2-type immune response, proliferation and cytokine responses by splenic T cells from oral allergen- or placebo-challenged mice were determined (Fig. 1 *E–H*). Allergen stimulation induced a significant proliferation response in the oral allergen-challenged mice but not the control group (Fig. 1*E*). In contrast, the proliferation responses of T cells to nonspecific polyclonal activation (αCD3/αCD28) was comparable between placebo- and oral allergen-challenged mice (Fig. 1*E*). To characterize the phenotype of the allergen-reactive T cells, IL-4, IL-5, and IFNγ levels in the splenocyte cultures were measured (Fig. 1 *F–H*). The addition of OVA to the T cells of oral allergen-challenged mice induced the production of IL-4 and IL-5, but no detectable IFNγ, a cytokine produced by Th_1 cells. In contrast, splenocytes isolated from placebo-challenged mice produced only low levels of IL-5 and no detectable IL-4 or IFNγ (Fig. 1 *F–H*). Collectively, these data indicated that oral allergen challenge promoted the expansion of a Th_2-biased immune response.

Histological examination of the jejunum revealed vascular congestion, edema, and a prominent cellular infiltrate in the oral allergen-challenged mice as compared with placebo-challenged mice (Fig. 2 *A* and *B*). The cellular infiltrate was predominantly localized to the mucosa and lamina propria throughout the small intestine and was primarily composed of eosinophils (Fig. 2*B*, and results not shown). Elevated levels of eosinophils were also observed in the duodenum and ileum of oral allergen-challenged mice as compared with placebo-challenged mice (results not shown). The presence of eosinophils was further characterized by standard immunohistochemical analysis by using an antiserum specific for eosinophils (anti-MBP) (Fig. 2 *C* and *D*). The infiltrating eosinophils were observed interspersed throughout the reticular connective tissue of the lamina propria and mucosa and throughout the length of the lamina propria of the villi (Fig. 2 *B* and *D*). Placebo-challenged mice had low levels of eosinophils predominantly localized to the base of the villus in the region of the crypt of Lieberkühn and occasional cells within the lamina propria of the villus (Fig. 2 *A* and *C*). This distribution is similar to the location of eosinophils at baseline (naïve mice), indicating that placebo challenge alone did not significantly affect eosinophil trafficking (18). Morphometric analysis revealed that the level of eosinophils in oral allergen-challenged mice was significantly higher ($P < 0.0001$) than that observed in placebo-challenged mice (Fig. 3). Because mast cells have also been implicated in the pathogenesis of various gastrointestinal hypersensitivity responses (11, 13), we were interested in examining their participation in oral antigen-induced eosinophil gastrointestinal allergy. Histological analysis of the jejenum revealed no significant difference in the level of mast cells within the lamina propria and villus between placebo-challenged and oral allergen-challenged mice (results not shown).

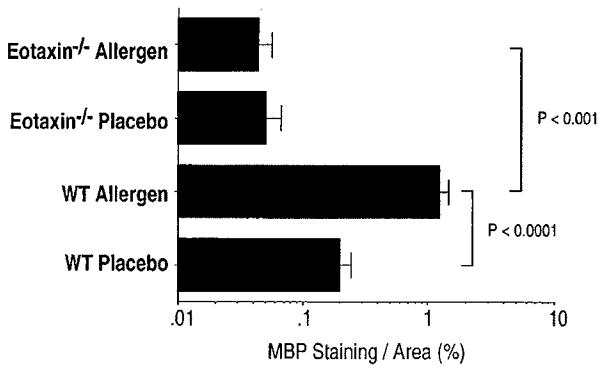

Fig. 3. Quantification of gastrointestinal eosinophils by morphometric analysis in placebo- and oral allergen-challenged wild-type and eotaxin-deficient mice. Gastrointestinal eosinophil numbers were quantified in placebo- and oral allergen-challenged wild-type (WT) and eotaxin-deficient (eotaxin$^{-/-}$) mice by morphometric analysis. Data were obtained on day 19. Data represent the mean ± SEM of four to five random sections/mouse for four to five animals per group and are representative of three separate experiments. Statistical significance of experimental groups was analyzed by using the unpaired Student's *t* test.

Critical Role of Eotaxin in Antigen-Induced Experimental Eosinophilic Gastrointestinal Allergy. Elevated levels of eotaxin and eosinophils have been associated with various human inflammatory disorders, and increased levels correlate with disease severity (26, 27). Thus, it was critical to determine the role of this chemokine and gastrointestinal eosinophils in gastrointestinal allergic inflammation. Oral allergen challenge of eotaxin-deficient mice induced a marked increase in peripheral blood eosinophils compared with placebo-challenged eotaxin-deficient mice (Fig. 1*B*). Interestingly, the peripheral blood eosinophilia was significantly higher than that of oral allergen-challenged wild-type mice (Fig. 1*B*). We hypothesized that the elevated level of eosinophils in the peripheral blood of oral allergen-challenged eotaxin-deficient mice was because of failure to recruit eosinophils to the gastrointestinal tract, thus preventing the transmigration of circulating eosinophils into the site of inflammation. To test this hypothesis, we examined the level of eosinophils in the lamina propria of the small intestine of oral allergen-challenged eotaxin-deficient mice. Histological analysis of the intestinal tissue from oral allergen-challenged mice revealed no significant morphological changes to the small intestine structural integrity in the absence of eotaxin (Fig. 2*F*; data not shown). Morphometric analysis of anti-MBP-stained tissue revealed that in contrast to wild-type mice, oral allergen challenge of eotaxin-deficient animals induced no significant increase in the level of eosinophils as compared with placebo-challenged eotaxin-deficient mice (Fig. 3). The level of eosinophils in oral allergen-challenged eotaxin-deficient mice was markedly reduced compared with wild-type mice ($P < 0.001$) (Fig. 3). This indicates that the reduced baseline level of gastrointestinal lamina propria eosinophils in eotaxin-deficient mice is not increased by allergen challenge (28). The reduction of intestinal inflammation in the absence of eotaxin was not because of the failure to develop allergen-specific lymphocyte responses, because eotaxin-deficient mice produced marked levels of allergen-specific IgE and IgG_1 (Fig. 1 *C* and *D*) and Th_2 cytokines (data not shown).

Normal Levels of CCR-3 and $\alpha_4\beta_7$ in the Absence of Eotaxin. We next examined the mechanism for the impaired recruitment of eosinophils into the gastrointestinal tract in the absence of eotaxin. Recent investigations have demonstrated that other chemokines, including RANTES, may induce eosinophil chemotaxis via the eotaxin receptor, CC-chemokine receptor 3 (CCR3) (6, 29, 30). To determine whether ablation of eosinophil transmigration was specific for

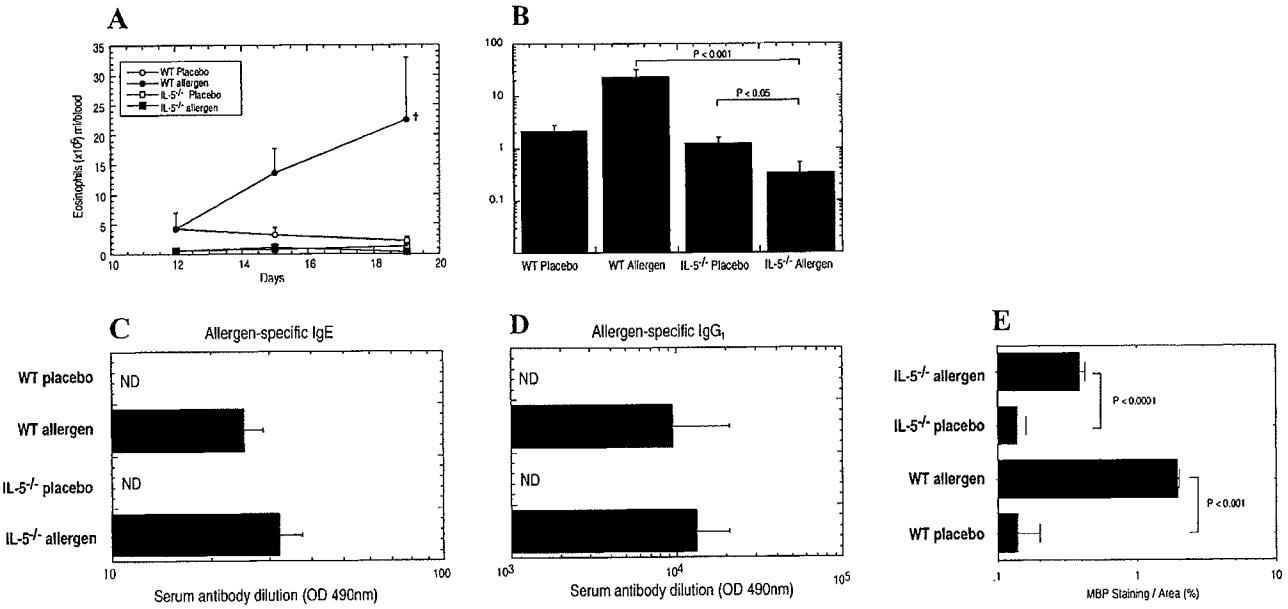

Fig. 4. Gastrointestinal allergy in IL-5-deficient mice. (*A*) Eosinophil numbers in the peripheral blood of placebo- and allergen-challenged wild-type and IL-5-deficient mice. Eosinophil numbers in the peripheral blood of placebo- and allergen-challenged wild-type and IL-5-deficient mice on days 12, 15, and 19 are shown. (*B*) Eosinophil numbers in the peripheral blood of placebo- and allergen-challenged wild-type and IL-5-deficient mice on day 19. (*C* and *D*) The level of allergen-specific IgE (*C*) and IgG_1 (*D*) in serum of placebo- and allergen-challenged wild-type and IL-5-deficient mice. (*E*) Eosinophil numbers in the jejunum were quantified by morphometric analysis. Data represent the mean ± SEM for (*A–E*) SEM of four to five random sections/mouse for four to five animals per group and are representative of two separate experiments. Statistical significance of experimental groups was analyzed by using the unpaired Student's *t* test (*A*). +, $P < 0.01$ compared with placebo-challenged wild-type (WT) mice.

eotaxin and was not because of a loss of CCR3, we examined the level of expression of CCR3 on peripheral blood eosinophils from oral allergen-challenged wild-type and eotaxin-deficient mice. We observed no difference in the level of CCR3 expression between wild-type and eotaxin-deficient mice, suggesting that eotaxin was not required for CCR3 expression [the mean fluorescence intensity for CCR3 was 23.8 and 21.8 for eosinophils from wild-type and eotaxin-deficient mice, respectively; see supplementary data (www.pnas.org)]. We also examined the level of expression of the eosinophil adhesion molecule, $\alpha_4\beta_7$, because this integrin has been shown to be responsible for the recruitment of leukocytes into the intestinal lamina propria and associated lymphatic organs (31, 32). Eosinophils from the peripheral blood of oral allergen-challenged wild-type mice expressed $\alpha_4\beta_7$ at the same level as eosinophils from eotaxin-deficient allergen-challenged mice (the mean fluorescence intensity for $\alpha_4\beta_7$ was 8.56 and 9.82 for eosinophils from wild-type and eotaxin-deficient mice, respectively; see supplementary data). Collectively, these results suggest that the failure to recruit eosinophils into the intestine of allergen-challenged mice is most likely because of loss of the eotaxin concentration gradient in the intestine.

The Role of IL-5 in Experimental Eosinophilic Gastrointestinal Allergy. We were next interested in determining the role of IL-5 in regulating eosinophil-associated gastrointestinal allergy because this cytokine is a pivotal modulator of eosinophil trafficking during allergic airways inflammation (8, 17, 33, 34). IL-5 has been shown to mobilize eosinophils from the bone marrow into the circulation and to promote eosinophil tissue trafficking during allergic airway inflammation (8, 35, 36). We therefore compared oral allergen-induced gastrointestinal allergy in IL-5-deficient and wild-type mice. In marked contrast to wild-type mice, allergen challenge of IL-5-deficient mice did not promote a peripheral blood eosinophilia (Fig. 4 *A* and *B*). Interestingly, after the second allergen challenge (day 19), the level of eosinophils in the blood of these mice was significantly lower than that of placebo-challenged IL-5-deficient mice ($P < 0.05$; Fig. 4*B*). Levels of allergen-specific IgE and IgG_1 resembled those present in wild-type mice (Fig. 4 *C* and *D*). These data suggest that the limited number of circulating eosinophils present in IL-5-deficient animals were being recruited into the intestine after allergen challenge, thereby depleting the level of peripheral blood eosinophils. To prove this, morphometric analysis of anti-MBP staining of IL-5-deficient mice revealed a 3-fold increase in the level of eosinophils recruited into the intestine after allergen challenge (Fig. 4*E*). The level of eosinophil recruitment was still lower than in allergen-challenged wild-type mice. These studies also indicated that the baseline production of eosinophils, which occurs independently of known eosinophil hematopoietins (IL-3, IL-5, and GM-CSF) (18), provides a sufficient number of eosinophils for the development of tissue eosinophilia.

Discussion

We have developed a model for oral allergen-induced eosinophil-associated gastrointestinal allergy that mimics a variety of human gastrointestinal allergic conditions. Although no murine model adequately mimics human disease, our experimental regime offers an experimental framework to analyze the events associated with antigen-induced eosinophil-associated gastrointestinal allergy. Our results establish that oral allergen is sufficient to promote the recruitment of eosinophils to the gastrointestinal tract. Interestingly, it was previously known that the level of gastrointestinal eosinophils in a subset of patients with eosinophilic gastrointestinal inflammation decreases after a specific food elimination diet, but no causal link between allergen sensitization and exposure and gastrointestinal eosinophil levels was established (11, 13). Importantly, we have identified eotaxin as a key regulator of eosinophil trafficking during gastrointestinal allergic processes. It has previously been shown that eotaxin has a nonobligatory role in regulating

allergic responses in the lung because eosinophil recruitment and airway hyperreactivity during the sustained late phase response developed in allergen-challenged eotaxin-deficient mice (9, 10). Furthermore, antibody neutralization studies with bronchoalveolar lavage fluid from humans with asthma have suggested only a partial role for eotaxin in the pathogenesis of asthma (26). In the present study, allergen-induced eosinophilic infiltration in the gastrointestinal tract was shown to be markedly impaired in the absence of eotaxin, suggesting that the mechanism for eosinophil homing into this mucosal tissue differs from that operational in the lung (9, 10).

Only a limited number of experimental systems have been reported to mimic eosinophilic gastrointestinal diseases. For example, in one system, anaphylaxis was induced in mice that were injected with hybridoma cells secreting anti-TNP IgE (37). These experimental systems, although reporting eosinophilic inflammation, have not examined the mechanisms involved in eosinophil trafficking or effector function. Several other gastrointestinal allergy models have been reported, but these mimic noneosinophilic gastrointestinal allergic disorders. For example, allergen treatment of mice passively sensitized with IgE results in mast cell-dependent neutrophil recruitment (38). Furthermore, sensitization with allergen and cholera toxin induced mast cell degranulation and anaphylaxis but no eosinophil recruitment into the gastrointestinal tract (39).

Our data also provide an explanation for the dichotomy that is often observed between peripheral blood and tissue eosinophilia in various diseases (40). For example, patients with gastroesophageal reflux have eosinophilia in the esophagus but rarely have elevated circulating eosinophil numbers, and only a subset of patients with asthma has peripheral blood eosinophilia. Furthermore, drug-induced eosinophilia is usually limited to the peripheral blood (40). The finding that peripheral blood and tissue eosinophilia can be dissociated in the absence of eotaxin indicates that the relative balance between the expression of eotaxin and eosinophil hematopoietins (e.g., IL-5) can have profound effects on the relative distribution of eosinophils. As a corollary, underexpression of IL-5 relative to eotaxin can lead to gastrointestinal tissue eosinophilia in the absence of circulating eosinophilia. This is supported by the finding that oral allergen challenge of mice deficient in IL-5 results in a decrease in the circulating level of eosinophils compared with wild-type mice.

In summary, this investigation provides insight into the molecular mechanisms involved in allergic responses of the gastrointestinal tract. We demonstrate that oral allergen challenge to sensitized mice promotes allergic inflammation characterized by Th2-associated eosinophil accumulation in the peripheral blood and small intestine. Additionally, we demonstrate the critical role of eotaxin in regulating allergen-induced eosinophil trafficking to the lamina propria of the gastrointestinal tract. Furthermore, we demonstrate that eosinophil recruitment to the gastrointestinal tract can occur in the absence of the major eosinophil growth factor IL-5. Lastly, we provide a molecular explanation to explain the dichotomy seen between peripheral blood and tissue eosinophilia in a variety of medical diseases. These data indicate that agents that block eotaxin and/or CCR3 may have beneficial effects on modulating eosinophil-associated gastrointestinal allergies. It is hopeful that this study will provide the necessary framework to examine allergic responses in the gastrointestinal tract with the same scrutiny that has been applied to the study of allergic processes in the lung.

We thank Connie Lobas for excellent technical assistance and F. Finkelman, M. Cohen, A. Srikiatkhachorn, G. K. Hershey, S. Wert, N. Zimmermann, and L. Poulos for critical reading of the manuscript and helpful discussions. We thank A. Lippelman for secretarial assistance and Alicia Emly for graphic assistance. We also thank Robert Coffman for the anti-CCR3 serum and James and Nancy Lee for anti-MBP serum. This work was supported in part by the National Health Medical Research Council (Australia), C. J. Martin Postdoctoral Fellowship (S.P.H.), the Jaffe Family Fund of the American Academy of Asthma, Allergy, and Immunology (S.P.H.), National Institutes of Health grant R01 AI45898–01 (M.E.R.), and the Human Frontier Science Program (M.E.R. and P.S.F.).

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Oral Tolerance: Animal Disease Models and Human Trials

Summary of Part V

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In this segment of the conference the possible use of oral tolerance induction as a therapeutic modality in humans was the focus of discussion. While it is fair to say that the record of success of this treatment approach is, at best, mixed, there are nevertheless reasons to hope that oral administration of antigens can eventually be a useful way of controlling inflammatory diseases, and we are gradually learning how this goal can be accomplished. Weiner and his colleagues provide an in-depth review of oral tolerance and how it may be used in the therapy of patients, and our aim here is to highlight and discuss certain observations that relate to the immune mechanisms that underlie the clinical usage of this approach.

We can start our commentary with a discussion of a well-executed and provocative study by Moldoveanu and her associates that very clearly shows that oral antigen administration *after* initial immunization does not reduce a subsequent booster response to the same antigen; in other words, oral antigen administration does not influence (downregulate) an already established response and, by extension, would not influence the ongoing response to self-antigens in autoimmune disease. These authors point out that this "negative" result mimics prior studies of "after-the-fact" oral tolerance induction in experimental animals, and, indeed, it fits with the fact that the success of oral tolerance induction in the treatment of experimental inflammation in rodents usually involves administration of the oral antigen at the time of initiation of disease, not after the disease has developed. It should be added that this result obtained by Moldoveanu can conceivably be explained by the studies of Strober and his colleagues (this volume, pages 115–131) who show that an ongoing Th1 response inhibits either the expansion of regulatory cells or the negative signaling by these cells.

These results clearly bode ill for reversal of disease by oral tolerance induction, which necessarily involves treatment of an already established immune response. However, several facts may mitigate this poor prognosis for this form of treatment. First, the study was done with an exogenous antigen (KLH) and thus may not lead to the stimulation of natural $CD25^+$ regulatory cells that have been shown to be specific for self-antigens. Thus, if the antigen selected for the induction of oral tolerance results in the expansion of already existent regulatory cells, it may have a

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Ann. N.Y. Acad. Sci. 1029: 310–312 (2004).
doi: 10.1196/annals.1309.078

greatly improved chance of success. It is important to remember here that the antigenic specificity of the regulatory cell does not have to be directed to the autoantigens actually causing tissue damage, only to the self-antigens present in the inflammatory site that can stimulate the regulatory cells to quell the inflammation by their bystander effects. This requirement for oral tolerance induction by a self-antigen may explain the interesting finding of Nussenblatt and his colleagues that a purified antigen had a better therapeutic effect in the treatment of uveitis by oral tolerance than did the mixture of antigens. In this situation, the limited number of antigenic epitopes of a purified antigen may mimic self-antigens and thus may better target regulatory cells than a mixture of antigens that may actually target effector cells and enhance cell injury.

A second fact to be considered in this context is that, as shown by Powrie and her colleagues (this volume, pages 132–141), administration of $CD25^+$ regulatory cells seemed able to overcome an ongoing inflammation, even though the latter was the robust inflammation seen in cell-transfer colitis. One factor in this success was again the fact that the stimulating antigens for the regulatory cells were probably the abundant self-antigens in the mucosal milieu. Another factor, however, may have been that in this situation the regulatory cells were operative in an immunodeficient host and thus may have been better able to expand because they were unhindered by homeostatic controls of lymphocyte expansion existent in a more normal host. This leads to the possibility that oral tolerance induction may be enhanced in conditioned patients that more readily allow regulatory cell expansion. Such conditioning may involve controlled immunoablative procedures, such as the use of anticytokines that cause apoptosis of Th1 T cell effector cell populations prior to the institution of oral tolerance induction. In addition, as suggested by Staines and his colleagues, it may also involve the use of regulatory cell "adjuvants" that allow for expansion of the latter in the face of Th1 inflammatory responses.

The studies reported here highlight the wide spectrum of possible diseases subject to treatment by oral tolerance. In studies of experimental mice, new data from Weiner and colleagues indicate that the field of possible use for this therapeutic modality must now be widened to include treatment of atherosclerosis, stroke, and Alzheimer disease, diseases not normally included within the category of autoimmune states. Indeed, from these data it is fair to say that all inflammations, not only autoimmune inflammations, may be subject to control by oral tolerance induction. One disease addressed in this section that is of particular interest is Crohn's disease, a Th1-mediated inflammation of the small and/or large intestine that is thought to be due to excess reactivity to antigens in the mucosal microflora (i.e., antigens that are equivalent to self-antigens). As shown by Kraus and his colleagues, there is some reason to believe that this disease is, in fact, associated with a defect in oral tolerance induction, although it is far from clear whether this is a primary or secondary effect. Interestingly, this study was performed with the same exogenous antigen, KLH, as used in the Moldoveanu study and thus may not reflect oral tolerance mediated by self-antigen–reactive cells. In any case, Ilan and his colleagues report promising therapeutic results by feeding patients a colonic antigen preparation in an uncontrolled clinical trial. This brings us back to the point made above, that it is possible that this positive result was obtained because in this situation one is administering antigen that does address the self-antigens in the intestinal milieu that are widely thought to be the causative antigens in this disease.

A further point emphasized by several of the authors of clinical studies is that not only the choice of antigen, but also the dose and timing of the antigen may be critical to the success of oral tolerance induction as a treatment of disease. From the work of Teitelbaum and his colleagues, for instance, we learn that oral glatiramer acetate (copolymer I) administration was an effective means of preventing experimental autoimmune encephalomyelitis in two rodent species and in primates. In addition, these authors reported that in the rodent model this treatment was effective in chronic disease, that is, after the disease had been already established. In view of these positive results in experimental animals, it was disappointing that oral glatiramer acetate administration was not effective in treating patients with MS in a large multicentered trial. A possible reason for this failure was suggested by the fact that in the animal studies, oral glatiramer acetate induced a Th2 response as well as cells that produced suppressive cytokines, such as TGF-β, whereas in the study of humans this was not the case. In view of this qualitatively different response in humans, it is possible that either the dose or timing of the treatment did not quite match that used in the animal studies and that further manipulation of how this material was given would lead to more positive results.

Finally, it is important to mention an exquisitely well-designed study of treatment of patients at risk for the development of diabetes with oral insulin conducted by Ergun-Longmire and colleagues. While the overall results of this double-blind, placebo-controlled study was generally negative, oral insulin did delay beta cell failure in a subset of patients: those with newly diagnosed disease who were given a low dose of insulin. These results deserve follow-up and again point out the role of dose in the effectiveness of oral tolerance induction. However, another point to emerge clearly from this work is that the therapy seemed to work early in the course of disease, again emphasizing that suppressor cell response induced by oral antigen may only be effective before the effector cell response becomes too overwhelming.

Overall, it seems apparent that these early studies of the use of oral tolerance induction to treat inflammation have shown some successes tucked among the several failures of this treatment. On this basis, additional studies that take into consideration the nature of the oral agent that is used, its dose, and its time with respect to disease will yield more positive results. In addition, the whole question of how to "condition" the patient to respond to treatment by oral tolerance induction needs to be more fully explored.